CCLXIII. THE CATATORULIN TEST FOR VITAMIN B_1

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THE catatorulin test, using avitaminous pigeon's brain, may still occasionally prove useful, because it is fairly sensitive, reacting to less than $0{\cdot}2\gamma$ vitamin B_1, and because it has a low sensitivity to the phosphoric esters [Peters, 1937]. With the newest thiochrome test [Jansen & Westenbrink, 1938] small amounts of vitamin B_1 are evidently determined, but the Schopfer mould test does not distinguish between the different forms of vitamin [Sinclair, 1937], and the new yeast enzyme test does not separate the free vitamin from its monophosphoric ester. Further there are points of general application in the use of a tissue preparation of this nature for an enzyme test in which it is found that fine grinding or extraction of the tissue leads to loss of activity [McGowan & Peters, 1937]. For these reasons the collective experience of some years with this test is here recorded briefly; in particular, it is believed that a satisfactory theoretical basis has been found for the use of pyruvate and that the conditions have now been defined which make for the maximum degree of sensitivity.

The first catatorulin tests for vitamin B_1 using avitaminous pigeon's brain were based upon the increase in O_2 uptake observed in lactate solution as the result of adding the vitamin [Passmore *et al.* 1933]. This was extended to lactate and pyrophosphate [Kinnersley *et al.* 1935], when it was found that this gave larger differences. Finally it became clear that pyruvate rather than lactate was the essential substrate; attention was therefore diverted to pyruvate [Ogston & Peters, 1936], especially in view of the further increase in the O_2 differences which was thus obtained. At the same time these last changes meant giving up an equation which had served well previously with lactate for the 2nd hour of respiration. Recent papers from this laboratory [Peters, 1936; McGowan & Peters, 1937] show clearly the incorrectness of the theoretical basis of this Michaelis equation in which there was postulated a reversible equilibrium between vitamin B_1 and some essential component of the system. Now we know that a quite different series of reactions is involved. In the absence of vitamin B_1, some component (probably protein) of the pyruvate-oxidase system becomes unstable; this is proved by the fact that the effect of vitamin B_1 tends to be that of maintenance [Peters *et al.* 1935; Westenbrink & Polak, 1937]; the control decreases in respiration rate more rapidly than the sample containing vitamin, and also the washed avitaminous brain tissue gives a poor catatorulin effect which is not improved by the addition of the washings. The steadier values obtained for respiration during the second hour are only in part due to the removal of the residual substrates; they are probably associated with the inactivation of the unstable component of the system; the rate of inactivation increases with rising temperature. In lactate the series of changes summarized by the previous equation is complicated:[1] (*a*) lactate is converted into pyruvate; (*b*) vitamin B_1

[1] Another possible explanation of the catatorulin effect might be that added aneurin merely prevents the dissociation of the last traces of pyrophosphoric ester, but this is not consistent with the trifling effect of vitamin upon the normal brain.

reaches its unstable component by diffusion; (*c*) the vitamin may first have to be phosphorylated, a question which is not yet decided [Peters, 1937; Ochoa & Peters, 1938]. By using pyruvate (*a*) is eliminated.

In arriving at a new theoretical treatment for pyruvate as substrate it must be remembered that the Q_{O_2} observed has three components, (1) residual respiration (not due to pyruvate), (2) residual pyruvate respiration (due to residual traces of vitamin B_1) and (3) catatorulin respiration, due to added vitamin. We may call (1)+(2) *R*. If we assume that the added aneurin *a* acts either as a prosthetic group or coenzyme to some protein, *p*, we can write *ap* as the actual enzyme system. At any time the catatorulin O_2 uptake in the absence of complicating factors depends upon *ap*. Since *p* is very unstable at 38° in absence of aneurin and substrate [Peters, 1936], at any time after 30 min. no more *ap* can be formed; after this time therefore

$$Q_{O_2} \text{ observed} = k\,(R + ap).$$

We should expect the difference between the Q_{O_2} with and without added vitamin to remain constant after the 30 min. period, if the complete system is reasonably stable, because the diminution of the residual respiration (1) will be the same for each, and the residual traces of the pyruvate-oxidase system will remain equally stable. The curves shown in Fig. 1 are consistent with this view, which has been previously expressed.

For the period of respiration ½–2 hr. there is a fairly constant change produced by the aneurin in μl./g./hr., though the general level of respiration is falling. Taking recent experiments consecutively, I have calculated in 28 cases the average variation from the mean over the period 1–2 hr. for the separate half-hours, and have obtained the value $\pm 7{\cdot}8\ \mu$l.; this is only a small percentage of the total values involved. For the period 0·5–1·0 hr. the average difference from the same mean is greater, namely $\pm 15{\cdot}2\ \mu$l. Hence there is experimental justification for the idea that the system once formed remains stable, and proof that the values for the period 1–2 hr. are more consistent (σ for individual figures varying from 300–600 μl. for 1–2 hr. is 22 and for 0·5–1·0 hr. 63).

Further support for the idea that the aneurin system once formed is irreversible is to be found in the failure to obtain substantial catatorulin effects with normal brain mentioned above, and the absence of a catatorulin action in solutions in which normal brain has been previously shaken.

Accepting the hypothesis that the rate of pyruvate oxidation is proportional to the quantity *ap*, theoretically this rate should be exactly proportional to the amount of aneurin added, provided that the active surface adsorbs the aneurin practically quantitatively. Hence, up to a certain limit which is set by the amount of the residual aneurin system still in the avitaminous tissue, increases in rate due to equal increments of aneurin should be equal. There should be a straight line relation between the amount of vitamin and the catatorulin O_2 up to a maximum, which should then at once show no further increase. Fig. 2 (Theory) indicates the kind of curves which would be obtained if this view were correct. The typical experimental curves also shown in Fig. 2 are in general agreement with the hypothesis; they indicate that after the addition of 1·0γ there is in all cases an abrupt discontinuity; in many cases there is practically no increase after 0·5γ. This is consistent with slight variations in the residual vitamin left in different brains and it means that in practice the part of the curve from 0·5–1·0γ is dangerous to use for any but the most approximate estimations. Inspection of the experimental curves obtained during the last few years showed

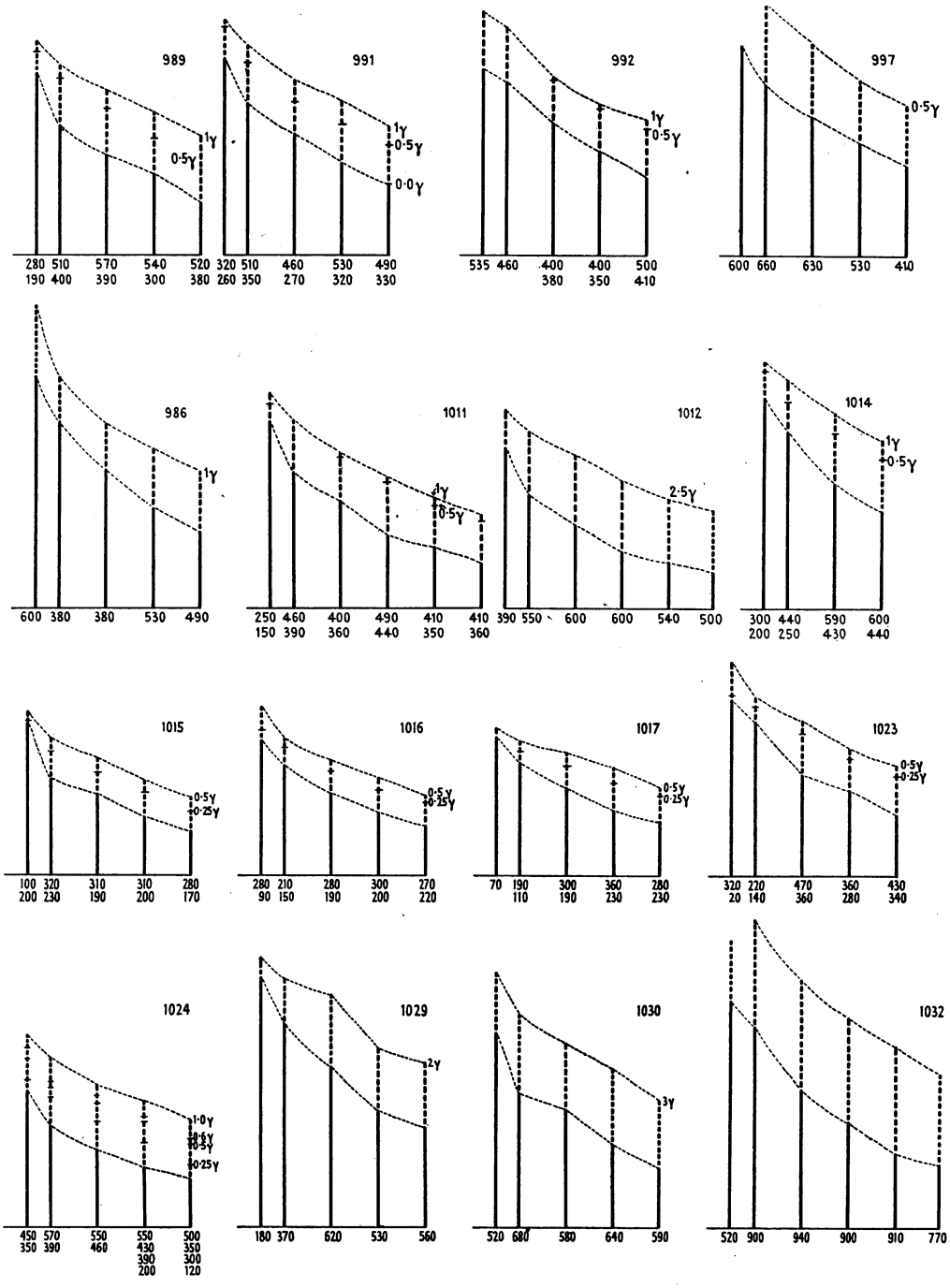

Fig. 1. Catatorulin tests indicating the variation of the respiration rate with time and different amounts of vitamin, indicated as γ on the diagram. Ordinate = rate of respiration, abscissa = time. Each vertical line represents the rate μl./g./hr. of respiration for successive periods, 15, 15, 30, 30, 30 min. The figures below the vertical lines represent the extra O_2 uptake due to vitamin.

several which departed more widely from the hypothesis above than could be attributed to experimental error. This departure is always in the sense that the 0·25γ point is higher than it should be for the straight line relation. Owing to the difficulty of standardizing conditions exactly in this system, it is not worth pursuing this point far; the best values for the catatorulin test will be obtained

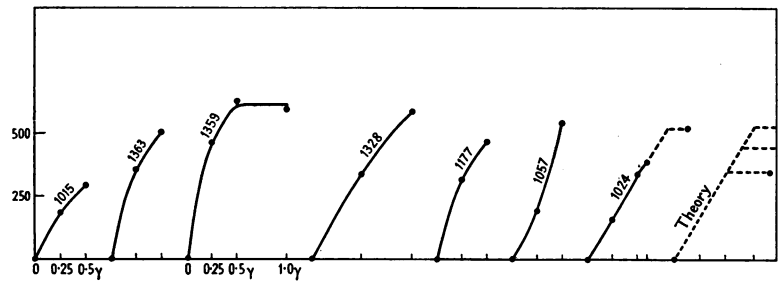

Fig. 2. Curves indicate the change in extra O_2 uptake produced by vitamin in varying concentration. Ordinate = O_2 uptake μl./g./hr.; abscissa = concentration of vitamin; the actual amount is indicated in two experiments; the remainder are drawn to the same scale. Last diagram marked "Theory" is drawn from theoretical considerations.

between 0·25 and 0·5γ, in the region of 0·3γ. I have found, however, that large differences are caused in catatorulin values by not allowing sufficient time for diffusion equilibrium to occur before introducing the bottles into the warm bath; an extreme example of this effect is given in Table I.

Table I. *Effect of allowing time for diffusion on the catatorulin test*

After division of the tissue at the mashing stage, the vitamin was immediately introduced into the bottles. Samples marked "No standing" were quickly filled with O_2 and placed in bath; those "Stood 15 min." were kept at room temperature for this time with shaking.

Extra O_2 uptake due to vitamin for period 1–2 hr., μl./g./hr.

Vitamin added	0·25γ	0·5γ
No standing	556	620
Stood 15 min.	343	523

Here the effect of standing has been to reduce the total O_2 uptake but to improve the divergence between the two concentrations of vitamin. The theoretical straight line is found when the latter values are plotted.

The improvements in method given below are based upon the above principles.

It is not easy to understand why the Michaelis type of equation, proposed by Passmore *et al.* [1933], held good for lactate. Peters *et al.* [1935][1] found that the pyruvate formed from lactate in presence of vitamin immediately underwent further oxidation. The most feasible explanation seems to be that the deciding factor was the instability of the pyruvate oxidase system in the absence of pyruvate.

Some experimental points

The procedure should follow that of Kinnersley *et al.* [1935] (Appendix, p. 712), with the following modifications. The birds should be thoroughly depleted of vitamin by dosing them at least once after symptoms have appeared and using them upon the reappearance of symptoms. Only the cerebrum and

[1] There was a misprint on page 66 of this paper. In Table II after the arrow in the heading, it should read "poison" addition instead of "substrate" addition.

optic lobes are used. The bottles should contain Ringer-phosphate solution at *p*H 7·3 and 6 mg. sodium pyruvate before the introduction of the samples. Tests showed that it made no difference whether the pyruvate was added before or after the mashing; it is more convenient to add it before. Triplicate estimations are made if possible, and there should be an attempt to get the points 0, 0·25 and 0·5γ with pure vitamin. The zero point can be obtained in duplicate if only a few samples are available. After division with the glass crusher, which should be carefully done, the vitamin should be added to form the last addition to the bottle soon after the crushing. In this way the concentration is not even temporarily larger than is intended. After the addition of vitamin the bottles should be allowed to stand at room temperature for about 10 min. before filling them with O_2 with sufficient shaking to ensure that they do not become anaerobic. An extra shake after filling them with O_2 is desirable. I have found it convenient to place the bottles in the warm bath at 38° at intervals of two each $\frac{1}{2}$ min.; this allows reasonable time for reading. The calculations are made by plotting the average for the extra O_2 uptake on squared paper for the period 1–2 hr. and reading the value for the unknown by inspection. As a convention I have usually joined the points 0, 0·25 and 0·5γ by straight lines when the 0·25γ point has not lain upon the direct line between 0 and 0·5γ. In some cases it would be unquestionably correct to continue a straight line through the points 0 and 0·25γ and assume that this becomes discontinuous at some point between 0·25 and 0·5γ; but the possibility that there is more initial vitamin than the equivalent of 0·5γ in the tissue has to be balanced in such cases against the possible error of determining the points 0 and 0·25γ in question. The standard deviation of a single observation is of the order of 3·5% of the mean. It is not always easy to get more than ten satisfactory samples from a given brain; the mixing of two avitaminous brains has not proved an advantage owing to the increasing standard deviation found for individual samples. Hence the improvement with triplicate estimations must be balanced against the fewer determinations possible. The theoretical error for an estimation using triplicate observations is of the order of $\pm 5\%$. It is difficult to give an exact idea of the error of the method in practice, because it is so much affected by the condition of the brain used; the results from some brains have to be discarded.

Exps. 1 and 2 below indicate the kind of results which may be expected and which are often obtained; but it must be emphasized that, as the method is subject to unaccountable variations, it is unwise to rely upon less than three estimations.

Exp. 1. Two tests were made in which 0·3γ of a vitamin solution was compared against 0·0, 0·25 and 0·5γ. The values found by estimation were 0·32 and 0·33γ.

Exp. 2. Two synthetic specimens of vitamin B_1, now known to be equally active, were tested at a level of 0·5γ. The mean O_2 uptakes during the period 1–2 hr. in duplicate estimations were in μl./g./hr.: specimen 1, with 0·0γ, 656 and with 0·5γ, 1027; specimen 2, with 0·5γ, 1063 and with 0·0γ, 654. The two separate values with 0·0γ agree well. The excess O_2 values of 371 and 409 give a value for specimen 2 of approx. 0·55γ. The extrapolation seems to be justifiable in this case; but the experiment was an early one, done before it was realized that 0·5γ was practically the maximum amount which could be estimated by this method.

The sodium pyruvate used was prepared as advised by Dr Stedman. I have found it best to dilute redistilled pyruvic acid with about an equal volume of water, add bromophenol blue and neutralize with concentrated pure NaOH to *p*H 4·5 approximately.

After cooling in ice-salt mixture, the salt is thrown out with acetone. Recrystallization was best done by dissolving in a minimum amount of water, treating with acetone until the appearance of strong turbidity, warming until the whole had just dissolved, and cooling in an ice-salt mixture. Purity was checked by estimation of the bisulphite-binding power.

SUMMARY

The catatorulin test using avitaminous pigeon brain has been studied further; it may still occasionally be useful owing to its specificity for small amounts of vitamin B_1 (about $0{\cdot}2\gamma$).

Several improvements have been introduced; pyruvate is substituted for lactate. An explanation has now been found for the discontinuous type of curve found with pyruvate and the upper limits of sensitivity have been defined.

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